RNA Interference Targeting VP1 Inhibits Foot-and-Mouth Disease Virus Replication in BHK-21 Cells and Suckling Mice

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RNA interference (RNAi) is a powerful tool to silence gene expression posttranscriptionally. In this study, we evaluated the antiviral potential of small interfering RNA (siRNA) targeting VP1 of foot-and-mouth disease virus (FMDV), which is essential during the life cycle of the virus and plays a key role in virus attachment to susceptible cells. We investigated in vivo the inhibitory effect of VP1-specific siRNAs on FMDV replication in BHK-21 cells and suckling mice, a commonly used small animal model. The results showed that transfection of siRNA-expressing plasmids gave an 80 to 90% reduction in the expression of FMDV VP1 in BHK-21 cells. Moreover, BHK-21 cells transiently transfected with siRNA-expressing plasmids were specifically resistant to FMDV infection when exposed to 100 50% tissue culture infective doses of virus, and the antiviral effects extended to almost 48 h postinfection. Furthermore, subcutaneous injection of siRNA-expressing plasmids in the neck made suckling mice significantly less susceptible to FMDV. In conclusion, our data suggests that RNAi may provide a viable therapeutic approach to treat FMDV infection.

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of cloven-hoofed animals (31). The etiological agent of FMD is FMD virus (FMDV), which belongs to the genus *Aphthovirus* of the family *Picornaviridae* (24). Current FMD vaccines based on inactivated virus are effective in preventing the disease but present the risks of incomplete inactivation or escape of virus from vaccine production laboratories (18). The development of recombinant peptide vaccine and chemically synthetic vaccine has achieved great success, as reported previously (14, 39). Although these vaccines are safe and effective in eliciting antiviral activity, they fail to induce immune response in a short period. Thus, the development of emergency antiviral strategies is necessary in order to prevent outbreaks of FMD.

RNA interference (RNAi) is the process of sequence-specific, posttranscriptional gene silencing (PTGS) in animals and plants, which is induced by 21- to 23-nucleotide (nt) small interfering RNA (siRNA) that is homologous in sequence to the silenced gene (40, 44). RNAi not only regulates gene expression in mammalian cells but also acts as a cellular defense mechanism against invaders, including viruses. siRNA has demonstrated antiviral potential in certain cultured mammalian cells and animals (15, 33). Therefore, 21- to 23-nt siRNA duplexes provide a new tool for gene-specific therapeutics for viral disease. siRNA can be synthesized by chemical methods or by in vitro transcription (43, 44). Recently, a DNA vector-based RNAi technology has been developed and has highlighted the general utility of RNAi against viruses in vivo (35).

The FMDV genome is composed of a positive-stranded RNA molecule of about 8,500 nt containing a unique open reading frame. The open reading frame encodes four structural proteins (VP1, VP2, VP3, and VP4), of which VP1 is essential during the life cycle of the virus and plays a key role in virus attachment to susceptible cells (1, 2). The prominent G-H loop of VP1 capsid protein of FMDV contains the evolutionarily conserved RGD tripeptide, which is a well-known ligand for specific cell surface integrins, including the $\alpha_5\beta_1$ receptor for fibronectin (26), the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors for vitronectin (30), and the $\alpha_{IIb}\beta_3$ receptor for fibrinogen (25). Competition assays with synthetic peptides suggest that the carboxy terminus of VP1 protein, which is located in the vicinity of the RGD motif, is necessary for RGD-mediated cell binding (5).

Here we report that a DNA vector-based RNAi technology specifically suppresses the expression of FMDV VP1 in BHK-21 cells and inhibits FMDV replication in BHK-21 cells and suckling mice.

MATERIALS AND METHODS

Cells, animals, and viruses. BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (pH 7.4). Cultures were incubated at 37°C with 5% CO_2. Suckling mice (C57BL/6), 2 to 3 days old and weighing 3 to 4 g, were purchased from the Institute of Biotechnology, Zhejiang Academy of Agricultural Sciences. Two FMDV isolates of serotype O (HKN/2002 [GenBank accession number AF525458] and CHA/99 [GenBank accession number AJ318833]) and one pseudorabies virus (PRV) isolate (Ea [GenBank accession number AY318876]) were used for viral challenge.

Construction of plasmids. The mouse U6 promoter was chemically synthesized according to GenBank sequence data (accession number X06980) and cloned into pcDNA3.1B(−) vector (Invitrogen, Groningen, The Netherlands), replacing the cytomegalovirus promoter in it, to generate the parent vector pU6. As a general strategy for constructing siRNA-expressing plasmids, inverted repeats targeting the mRNA of FMDV VP1 were subcloned into pU6 under control of the U6 promoter and termination signals of four or five thymidines (Fig. 1A). Plasmid pNT21 contains an inverted repeat corresponding to nt 16 to

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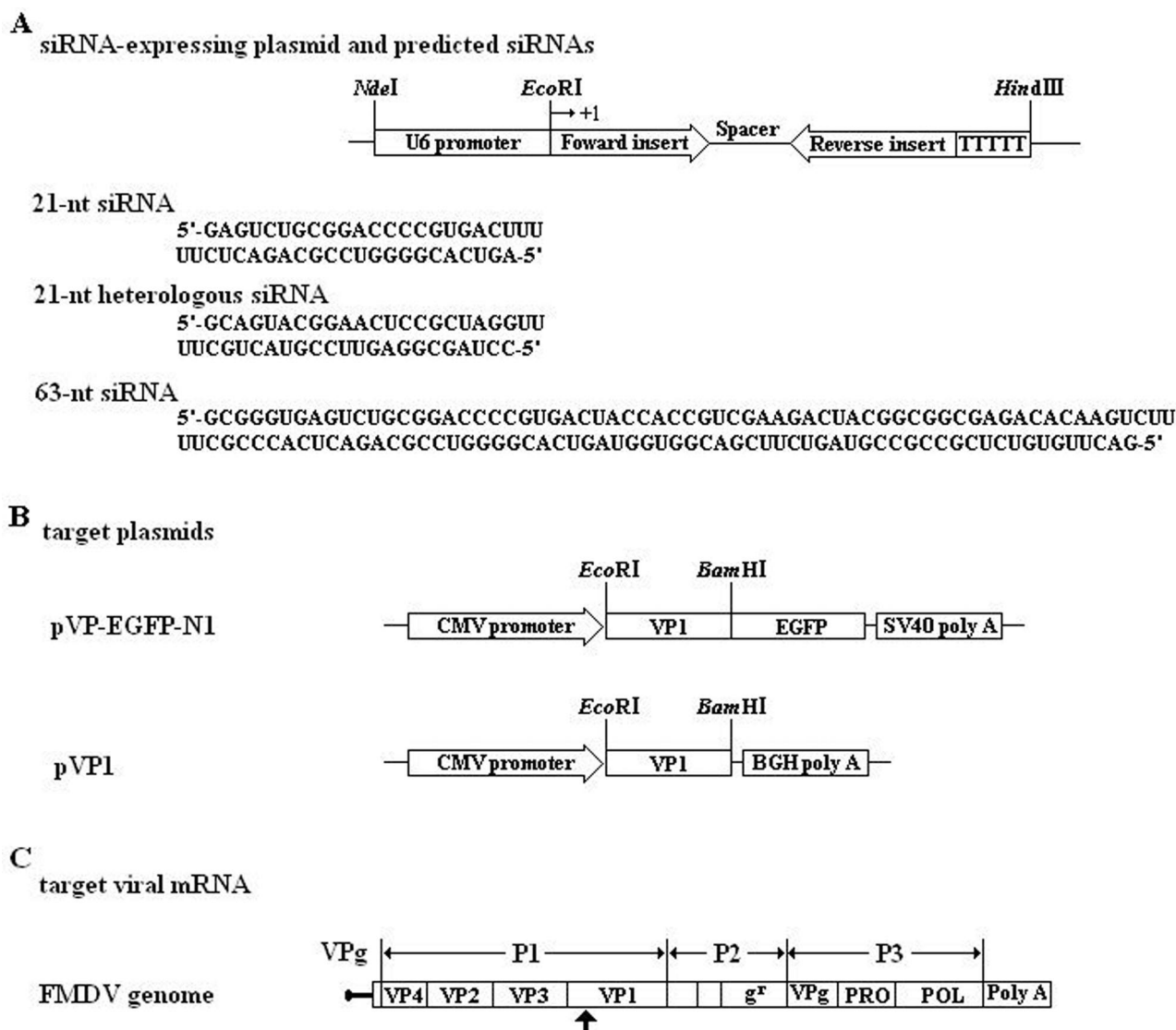
A siRNA-expressing plasmid and predicted siRNAs

21-nt siRNA
5'-GAGUCUGCGGACCCCGUGACUUU
UUCUCAGACGCCUGGGGCACUGA-5'

21-nt heterologous siRNA
5'-GCAGUACGGAACUCCGCUAGGUU
UUCGUCAUGCCUUGAGGCGAUCC-5'

63-nt siRNA
5'-GCGGGUGAGUCUGCGGACCCCGUGACUACCACCGUCGAAGACUACGGCGGCGAGACACAAGUCUU
UUCGCCCACUCAGACGCCUGGGGCACUGAUGGUGGCAGCUUCUGAUGCCGCCGCUCUGUGUUCAG-5'

B target plasmids

pVP-EGFP-N1

pVP1

C target viral mRNA

FMDV genome

FIG. 1. Schematic representations of siRNA-expressing plasmids, target constructs, target viral mRNA, and predicted siRNAs. (A) An inverted repeat is inserted at the 3' end of mouse the U6 promoter. The forward sequence of the repeat is 21 or 63 nt long, corresponding to the region of interest of the VP1 gene. The forward and reverse motifs are separated by a 6-nt spacer, 5'-CTCGAG-3'. The transcriptional termination signal of five Ts is added at the 3' end of the inverted repeat. The synthesized RNA is predicted to fold back to form a hairpin dsRNA, which would be finally processed into the putative siRNAs. (B) To monitor the function of siRNAs, the cDNA of HKN/2002 VP1 was cloned into pEGFP-N1 and pcDNA3.1B(−) vectors as described in Materials and Methods. (C) The FMDV genome contains a unique open reading frame. The arrow at the bottom shows the site targeted by VP1-specific siRNAs.

36 of the cDNA of HKN/2002 VP1, while plasmid pNT63 contains an inverted repeat corresponding to nt 10 to 72 of the cDNA of HKN/2002 VP1 (Fig. 1C). As a negative control for nonspecific effects, plasmid pNTH21 contains an inverted repeat of 21 nt heterologous to the HKN/2002 genome. For construction of target plasmids, pVP1 and pVP-EGFP-N1 were created. The cDNA (639 nt) of FMDV VP1 was obtained by reverse transcription-PCR (RT-PCR) amplification from an HKN/2002 RNA extract, using the primers 5'-CCGGAATT CATGACCACCTCTGCGG-3'(sense) and 5'-CGCGGATCCTCAAGAAGCT GTTTG-3' (antisense). Plasmid pVP1 was constructed by cloning the cDNA of FMDV VP1 into the EcoRI-BamHI sites of pcDNA3.1B(−), directly under the control of the cytomegalovirus promoter (Fig. 1B). To provide a reporting system for monitoring siRNA function, pVP-EGFP-N1 was constructed by cloning the cDNA of FMDV VP1 into the EcoRI-BamHI sites of pEGFP-N1 (Clontech, Palo Alto, Calif.) to fuse VP1 to enhanced green fluorescent protein (EGFP).

Transient cellular transfection. BHK-21 cells were transfected with plasmids either individually or in combination, using Lipofectamine 2000 (Invitrogen) as

directed by manufacturer. Cell monolayers grown in 96-well plates (about 80% confluent) were incubated for 5 h at 37°C in 5% CO_2 with 50 μl of DMEM containing plasmid DNA and Lipofectamine reagent complex. After transfection, 50 μl of growth medium containing twice the normal concentration of serum was added without removing the transfection mixture.

Analysis of VP1 and EGFP expression in BHK-21 cells. Cotransfection of target plasmids (0.2 μg) and either pU6 (0.1 μg) or siRNA-expressing plasmids (0.1 μg) was carried out at a 2:1 ratio with Lipofectamine 2000 as described by the manufacturer. After an additional 24 h of incubation, cells were examined microscopically for EGFP expression. Images were collected with an Olympus BH-2 microscope and a Nikon E950 video camera at a magnification of ×40 with an exposure time of 1/8 s.

Specific silencing of target genes was also confirmed by RT-PCR and sequencing. Total RNA extracts were prepared from transfected cells or controls with TRIzol reagent (GIBCO BRL) according to the manufacturer's protocol. To eliminate traces of DNA, samples were incubated for 1 h at 37°C with DNase

RQ1. To detect VP1 and EGFP mRNA expression in BHK-21 cells, 2 μg of RNA extracts was used as the template for RT-PCR amplification with the Superscript one-step RT-PCR system (GIBCO BRL). For retrotranscription of VP1 mRNA, the primers described above were employed. Primers for retrotranscription of EGFP mRNA were 5′-GCCACCATGGTGAGCAAG-3′ (sense) and 5′-CCCGCTTTACTTGTACAGC-3′ (antisense). RT-PCR products were further cloned into T-vector for sequencing.

Viral challenge assay in BHK-21 cells. Growth, isolation, and titration of viruses were all conducted with cultured BHK-21 cells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by using the Reed-Muench formula (27). A viral suspension titrated at 10^5 to 10^6 $TCID_{50}$/ml was used for the experiment. To assess the capacity of viruses to grow in BHK-21 cells expressing siRNAs, monolayers (about 80% confluent) of transiently transfected cells at 24 h posttransfection were grown in 96-well plates. Cells in one well of the 96-well plate were infected with 100 $TCID_{50}$ of virus per 0.1 ml. After 1 h of adsorption, the inoculum was removed and cells were washed twice with DMEM. The infection then proceeded in DMEM supplemented with 10% fetal bovine serum. Cells were examined microscopically, and images were collected with an Olympus BH-2 microscope and a Nikon E950 video camera at a magnification of ×40 with an exposure time of 1/8 s. Samples of supernatant were taken at different times postinfection (p.i.), and the virus titer ($TCID_{50}$) was determined three times on BHK-21 cells.

Viral challenge assay in suckling mice. Growth, isolation, and titration of viruses were all done with C57BL/6 suckling mice. The dose of FMDV was determined in four 10-fold serial dilutions of virus (i.e., 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}). Suckling mice (six groups of 10 each) were inoculated by subcutaneous injection in the neck with serially diluted viruses and then monitored for 5 days. The 50% lethal dose (LD_{50}) was estimated by the Reed-Muench method (27). The suckling mice were subcutaneously injected in the neck with 100 μg of plasmids dissolved in 100 μl of saline. After 6 h, the suckling mice were challenged with 20 or 100 LD_{50} of FMDV HKN/2002 per 0.1 ml by subcutaneous injection into the neck near the site which received the injected DNA and were observed for 5 days postchallenge. For detecting the therapeutic potential of siRNAs, some animals were treated with plasmid-virus complex and observed continually.

RESULTS

siRNAs synthesized in vivo specifically silence the VP1 gene of FMDV in BHK-21 cells. To evaluate the influence of siRNAs on the expression of FMDV VP1 in cultured cells, we cotransfected siRNA-expressing plasmids with either the target plasmid pVP-EGFP-N1 or the control plasmid pEGFP-N1 into BHK-21 cells. After 24 h of incubation posttransfection, we subjected the cells to fluorescence microscopic analyses and fluorescence-activated cell sorting. Cotransfection of either pNT21 or pNT63 with pVP-EGFP-N1 resulted in an 80 to 90% reduction in EFGP signal, relative to the control, whereas the control constructs (pU6 and pNTH21) gave no significant reduction of EGFP expression (Fig. 2A). The levels of inhibition mediated by the siRNAs were similar whether either a 21-nt siRNA or a 63-nt siRNA was expressed from the plasmid backbones. In contrast, cotransfection of pEGFP-N1 with any construct resulted in no significant reduction of EGFP expression compared with the control. The levels of inhibition of EGFP and VP1 mRNA expression were also demonstrated by RT-PCR analysis (Fig. 2C). The correct transcription of EGFP and VP1 was further confirmed by sequencing of RT-PCR products (data not shown). These results indicate that transient expression of siRNAs confers sequence-specific inhibition of the expression of FMDV VP1 in BHK-21 cells.

Transient expression of siRNAs confers specific resistance against FMDV in BHK-21 cells. A cell line derived from baby hamster kidney (BHK-21) has been extensively used for diagnosis and virus identification of FMDV (9). To study the effect of siRNAs expression on susceptibility to virus infection, trans-

fected cells (at 24 h posttransfection) were infected with 100 $TCID_{50}$ of FMDV HKN2002, FMDV CHA/99, or PRV Ea. BHK-21 cells are fibroblastic, grow in a monolayer, and have a well-defined tendency to parallel orientation (22). Viral infection causes a marked cytopathic effect (CPE) ending in total cellular detachment, rounding up, and destruction, which can be observed by microscopy (9). Microscopy examination revealed that the CPE on infected cells was delayed when the BHK-21 cells were transfected with siRNA-expressing plasmids (either pNT21 or pNT63), relative to the control (Fig. 3). However, cells transfected with either the parent plasmid (pU6) or a control plasmid (pNTH21) showed an extensive CPE within 24 h p.i. As expected, viral replication of FMDV CHA/99 or PRV Ea was not significantly altered in BHK-21 cells transfected with either siRNA-expressing plasmids or control constructs, suggesting that the antiviral effect mediated by siRNAs is efficient and highly sequence specific.

To further substantiate the antiviral activity, we determined the virus yield produced by cells infected with three viruses at 12, 24, and 48 h p.i. Less than 10^1 $TCID_{50}$ of HKN/2002 virus progeny was detected in supernatants collected from cells transfected with FMDV-specific siRNA-expressing plasmids at 12 h p.i., whereas over 10^4 $TCID_{50}$ of virus was determined in supernatants collected from control cells, pU6-transfected cells, or pNTH21-transfected cells (Fig. 4A). Transfected cells infected with CHA/99 or Ea did not show a significant reduction in virus yield at any time point assayed. The antiviral effects induced by either pNT21 or pNT63 were similar and extended to almost 48 h p.i. No significant inhibition was observed at 72 h p.i. The specificity of transient inhibition of virus yield was confirmed by the large amounts of virus progeny obtained when the sequence-divergent FMDV isolate CHA/99 and the unrelated PRV isolate Ea were included in the assay (Fig. 4B and C).

Treatment with siRNA-expressing plasmids rapidly induces an antiviral response in suckling mice. To test the potential anti-FMDV activity of the siRNAs, we challenged suckling mice pretreated by subcutaneous injection of siRNA-expressing plasmids in the neck or by of injection with plasmid-virus complex. All saline-treated ($n = 40$ mice per group) and pU6-treated ($n = 36$) mice died within 36 h, and most died within 30 h, after viral challenge (log-rank test, $P = 0.206$) (Fig. 5B). Mice ($n = 38$) treated with pNTH21, which was predicted to synthesize a 21-nt siRNA heterologous to the FMDV genome, were also not protected at all ($P = 0.19$). However, 31 of 40 mice pretreated with pNT21 and 29 of 40 mice pretreated with pNT63 survived a viral challenge of 20 LD_{50} for 5 days of observation. The difference between the survivals of mice treated with either pNT21 or pNT63 and the saline control was statistically significant ($P < 0.0001$ and $P < 0.0001$, respectively). In addition, animals treated with pNT21 (or pNT63)-FMDV complex or those pretreated with pNT21 but challenged with 100 LD_{50} of HKN/2002 also had reduced susceptibility to virus infection. A significant difference in mouse survival was also seen between mice treated with siRNA-expressing plasmids and controls ($P < 0.0001$) (Fig. 5A, C, and D). Internal organs from the surviving mice appeared normal when the animals were killed at the end of the observation period. All dead mice had extensive damage to the viscera, indicating massive virus replication in tested animals.
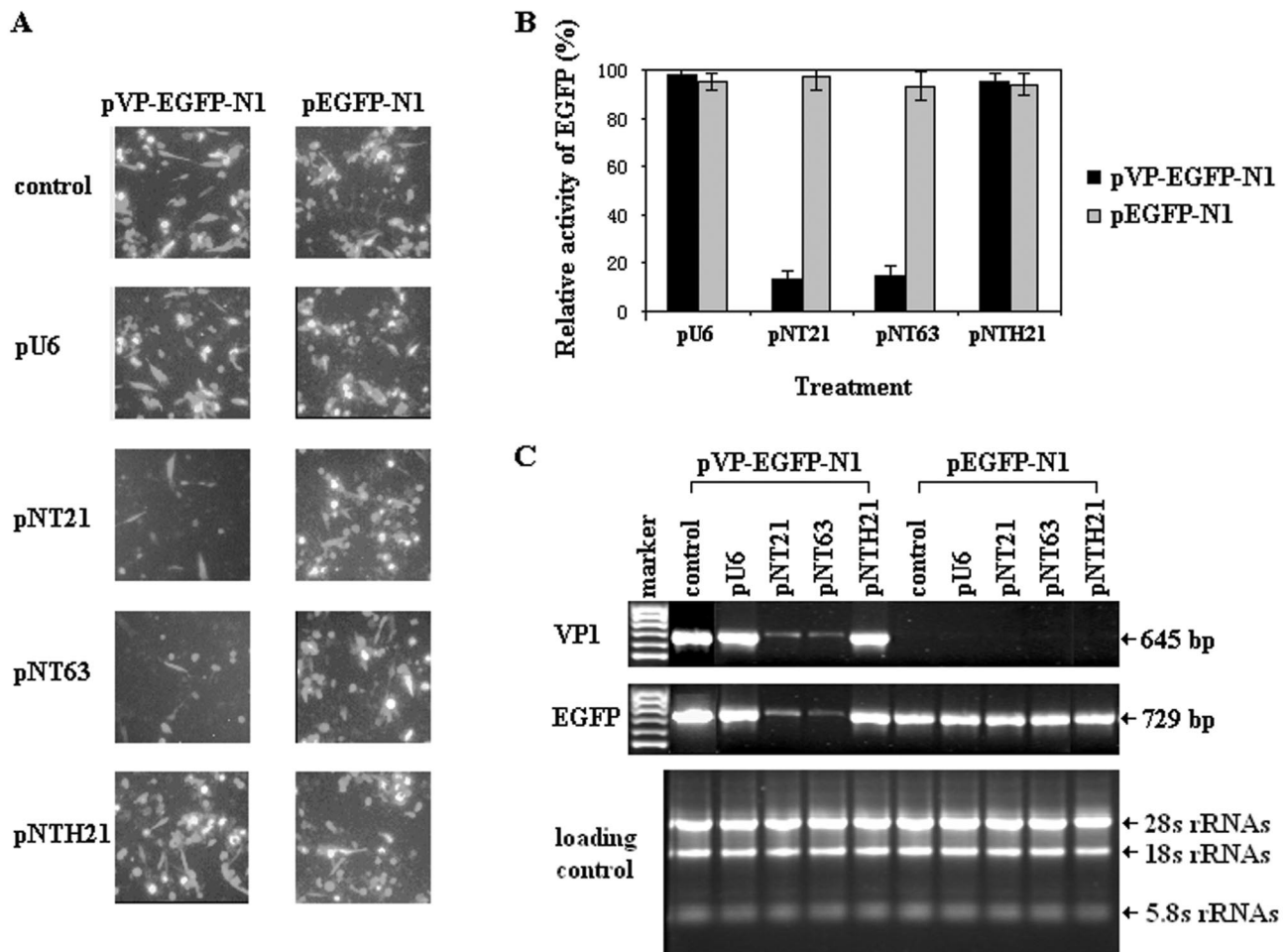
FIG. 2. Transient expression of siRNAs confers sequence-specific inhibition of expression of FMDV VP1 in BHK-21 cells. (A) Cells were cotransfected with pVP-EGFP-N1 and either pU6 or siRNA-expressing plasmids. As controls for nonspecific effects, cells were cotransfected with pEGFP and either pU6 or siRNA-expressing plasmids. Cells were visualized with a fluorescence microscope 24 h after transfection, and representative fields were photographed. (B) Inhibition of EGFP expression in cells cotransfected with pVP-EGFP-N1 (or pEGFP-N1) and siRNA-expressing plasmids. Cells were analyzed for EGFP expression by fluorescence-activated cell sorting, and the level of fluorescence relative to the control was quantitated. Data are the averages ± standard deviations from three separate experiments. (C) Cells were harvested, and transcripts were analyzed by RT-PCR amplification. The 28S, 18S, and 5.8S rRNAs were visualized under UV light for equal loading control.

To confirm FMDV inhibition, we performed FMDV-specific RT-PCR amplification and sequence analysis of RT-PCR products (data not shown). These experiments revealed a significant reduction (almost to the background level) in the products of VP1 mRNAs from siRNA-treated and surviving mice, whereas the VP1 transcript was not interfered with at all in control mice. Hence, siRNAs synthesized in vivo proved to efficiently inhibit FMDV replication in suckling mice.

DISCUSSION

In this study, we show that cotransfection of siRNA-expressing plasmids targeting specific sequences in VP1 of the FMDV genome results in a significant reduction in the corresponding viral transcripts. The targeted region in the viral RNA is essential during the life cycle of the virus. We designed two different anti-FMDV siRNAs, both of which proved to be remarkably effective and to similar extents. Expression of a 21-nt siRNA heterologous to the FMDV genome did not give a significant reduction of VP1 mRNA. In addition, RNAi action was abolished when targeted to a heterologous EGFP gene, suggesting that siRNAs mediated significant reductions in the levels of a specific target mRNA, and not a global down-regulation resulting from activation of the double-stranded RNA (dsRNA)-activated protein kinase R, which could lead to an inhibition of protein translation in a non-sequence-specific manner.

Most importantly, our results indicate that specific siRNAs dramatically inhibit viral replication in cultured cells that are susceptible to FMDV. It is well known that RNAi acts as a natural antiviral defense mechanism in plants, especially against RNA viruses (37). Mammalian cells were originally thought to be unlikely to posses an active RNA-silencing machinery (11) but primarily to induce a nonspecific, interferon-mediated antiviral response mediated by dsRNA (20, 34), especially by viral long (>35-nt) dsRNA (7). The recent
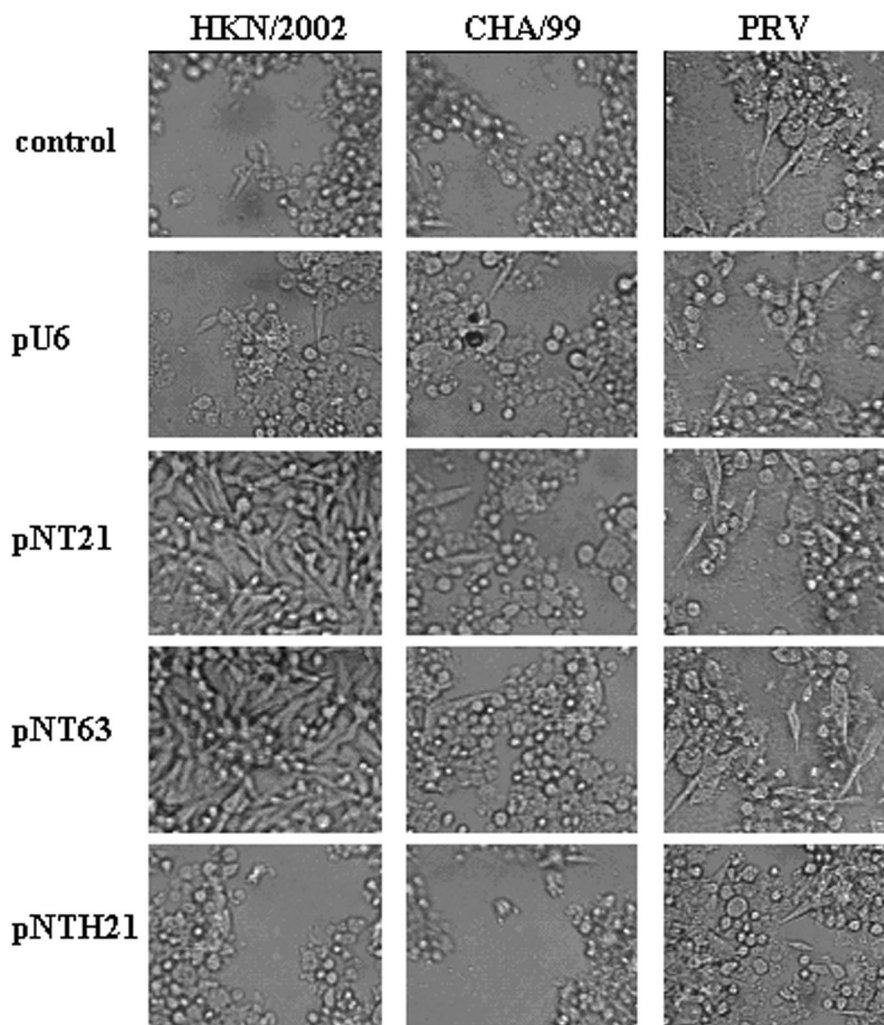
FIG. 3. Transient expression of siRNAs confers specific resistance against FMDV HKN/2002 in BHK-21 cells. Cells transfected with either pU6 or siRNA-expressing plasmids were infected with 100 TCID$_{50}$ of FMDV HKN/2002 per 0.1 ml. Cells were controlled by parallel assays with a divergent FMDV isolate (CHA/99) and an unrelated PRV isolate (Ea). After 24 h of infection, cells were visualized with an Olympus BH-2 microscope, and representative bright-field images were photographed.

description of RNAi in mammalian cells proved that the RNA-silencing machinery is conserved in mammals (10, 44). In some cases, long dsRNA was successfully processed into well-defined siRNAs and did not induce phosphorylation of protein kinase R, a signal of interferon-mediated activation of the innate defense system (6, 36). Surprisingly, a strong antiviral effect of RNAi was observed in the cases of human immunodeficiency virus (19, 23), hepatitis B virus (29, 33), and poliovirus and human papillomavirus (13, 16). Thus, although siRNA probably operates at multiple levels in mammals, its main action is expected to be mediated at the posttranscriptional level by rapid destruction of homologous mRNAs. Here we report that virus inhibition elicited by both a 21-nt siRNA and a long 63-nt siRNA is highly specific for FMDV and does not extend to an unrelated PRV or even a divergent isolate of FMDV, which shows nucleotide sequence similarities of 19 matches (21-nt siRNA) and 52 matches (63-nt siRNA). Further work should be done to screen for conserved targets for

RNAi along the viral genome, permitting the induction of cross-resistance to heterologous FMDV infection and to isolates from the seven different FMDV serotypes.

Strategies aimed at conferring rapid and efficient protection against FMDV have to face one main challenging factor: the rapid, acute infection caused by this virus, which makes the absence of sufficient amounts of antibodies or other interfering factors essential for protection. The traditional emergency vaccines based on virus inactivation could be effective in preventing disease within 4 to 5 days postvaccination, due to a critical role for innate immune defenses (3, 28). RNAi specifically targeting the viral transcripts severely impairs virus replication without activating nonspecific cellular responses, hence presumably minimizing undesirable side effects (33). Thus, antiviral strategies based on the specific and rapid inhibition of FMDV infection could complement and improve the traditional tools available to control this important animal pathogen. Our results indicate that treatment with FMDV-specific
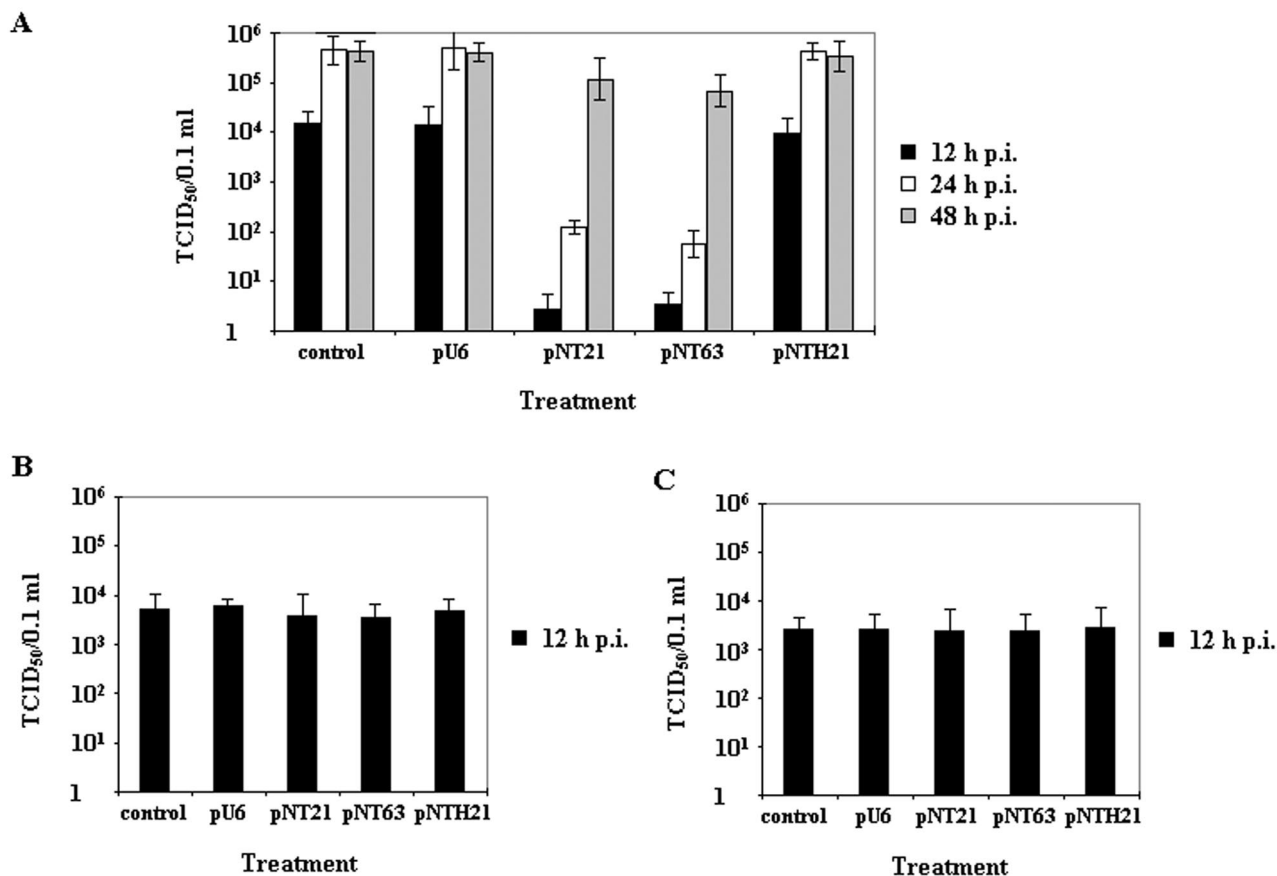
FIG. 4. BHK-21 cells transfected with pNT21 or pNT63 show a significant a reduction in virus yield, which is specific to HKN/2002 and extends to almost 48 h p.i. Cells transiently transfected with either pU6 or siRNA-expressing plasmids were infected with 100 TCID$_{50}$ of FMDV HKN/2002 (A), FMDV CHA/99 (B), or PRV Ea (C) per 0.1 ml, as described in Materials and Methods. Samples of supernatant were taken at different times p.i., and the virus titer (TCID$_{50}$) was determined three times on BHK-21 cells. Error bars indicate standard deviations.

siRNAs elicits an antiviral response in suckling mice within 24 h postchallenge. Especially, suckling mice treated with plasmid-FMDV complex have significantly reduced susceptibility to virus, suggesting the therapeutic promise of RNAi to prevent virus disease in animals.

Previous reports showed that the introduction of transgene-homologous DNA fragments initiated a systemic spread of sequence-specific PTGS in plants and could mediate PTGS targeted against virus (38). The results described above prompted us to explore the potential of the RNAi effect enhanced by preadministration of a transgenic plasmid encoding VP1 of FMDV before viral challenge. As expected, our additional experiment indicated that preadministration of the target plasmid pVP1 promoted survival of suckling mice after FMDV infection. The promoted antiviral effect was observed in suckling mice treated with both pNT21 and pVP1, showing about 80% survival, which is significantly higher than that of mice treated with pNT21 alone ($P < 0.0001$) (Fig. 6). To rule out inhibitory activity of pVP1 in a nonspecific manner, these animals were controlled by parallel assays either in the presence of pVP1 alone or in the presence of both pNTH21 and pVP1. No significant antiviral capability was exhibited in the parallel control assays ($P = 0.924$ and $P = 0.721$, respectively). It is well documented that siRNA amplification occurs in lower

species (17, 21), but this phenomenon is probably of little concern in mammals, because no RNA-dependent polymerase activity that would lead to amplification of an siRNA effect is seen in mammalian cells. There could be other mechanisms responsible for an enhanced RNAi effect in mammals. Previous studies demonstrate that the siRNA effect may be long lasting if viral mRNA is continually available, suggesting that whether siRNA is sustained or degraded could be determined by the presence or absence of target mRNA (32). Interestingly, our data also show that preadministration of the transgenic plasmid pVP1, which can express the FMDV VP1 transcript, contributes to the protection of animals. As reviewed previously (12), scientists predict the existence of a nucleic acid-based immune system at the beginning of life, by analogy to the protein-based immune system of mammals. If so, it is possible that an adaptive system that recognized foreign nucleic acids could be revived in mammals. It also stresses the fact that there is more to RNAi than we can yet fathom. Therefore, we suppose that siRNA-homologous mRNA produced from a transgenic vector presumably establishes a rudimentary form of memory, by analogy to the immunological memory in the protein-based immune system of mammals. More work will be performed to investigate this observation. Thus, our findings will probably promote vector-based RNAi technology.
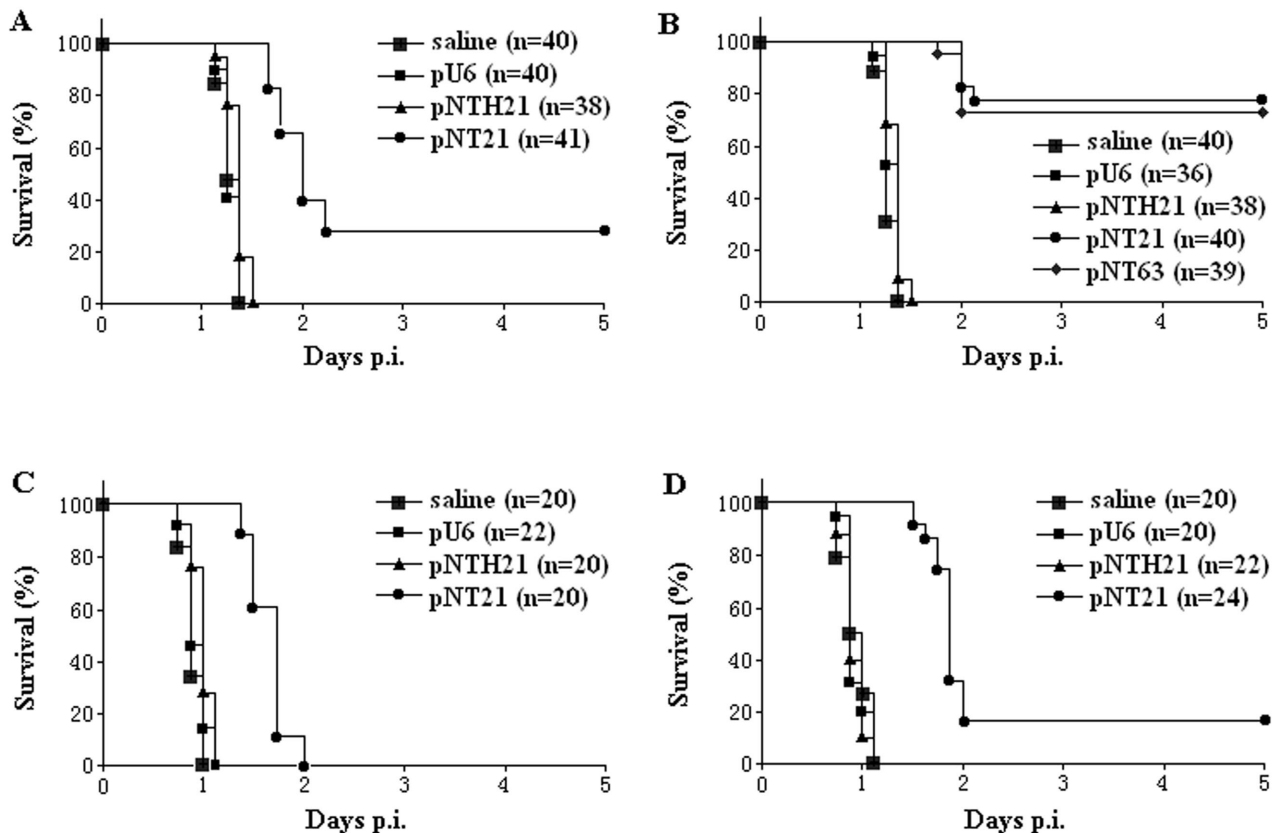
FIG. 5. siRNAs synthesized in vivo induce a rapid antiviral response in suckling mice and promote survival after FMDV infection. Suckling mice were challenged by subcutaneous injection in the neck with plasmid-virus complex containing 20 LD_{50} of HKN/2002 (A) or 100 LD_{50} of HKN/2002 (C). Alternatively, suckling mice were challenged by subcutaneous injection in the neck with 20 LD_{50} of HKN/2002 (B) or 100 LD_{50} of HKN/2002 (D) after 6 h of treatment with either pU6 or siRNA-expressing plasmids. All animals were observed for 5 days after challenge.
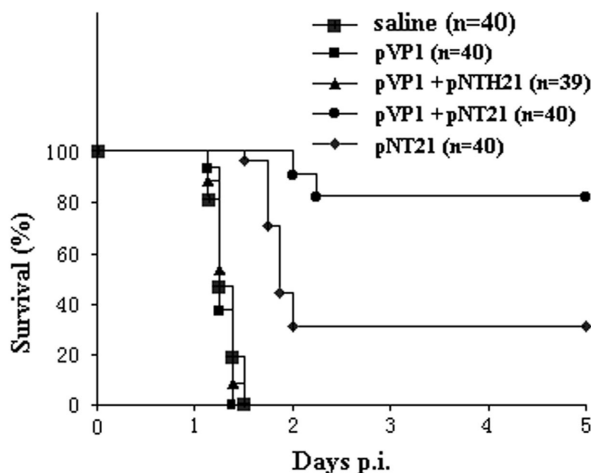
FIG. 6. Preadministration of pVP1 promotes mouse survival after FMDV infection. Suckling mice were challenged by subcutaneous injection with 20 LD_{50} of HKN/2002 after 6 h of treatment with 50 μg of pVP1 (or 50 μg of pNT21) or with a combination of pVP1 and pNT21 (or pVP1 and pNTH21) at the same dose and observed for 5 days.

The practical development of RNAi technology as a natural antiviral defense in mammals will require addressing at least three major issues. First, the RNAi effect in one infected cell should trigger a systemic antiviral response. In *Caenorhabditis elegans*, specific gene silencing induced by dsRNA injection in one region spreads to many different tissues, including the gonads (11). It is clear that a putative transmembrane protein, SID-1, accounts for the systemic RNAi in *C. elegans* (41). More excitingly, SID-1 homologues exist in nematodes, humans, and mice (41). Thus, although no direct evidence of systemic RNAi in mammals is found, more explorations should be conducted to investigate this phenomenon. In addition, viruses are likely to evolve mechanisms to suppress or escape an RNAi response. Focusing on the conserved regions of the viral genome which are sensitive to siRNA may be one approach to resolve this issue. Another alternative strategy is to target a single or several cellular factors involved in viral replication. Moreover, efficient delivery of siRNAs into cells or organs in vivo remains a major bottleneck in antiviral gene therapy. In our study, the simple injection of naked plasmid DNA encoding specific siRNAs proved to be effective in suckling mice, but the feasibility of this approach needs to be further evaluated in relevant species such as adult mice, guinea pigs, and even swine. Recent reports indicate that employing viral vectors will be a reasonable approach (4, 8, 42). In summary, it may be of great inter-

est to conduct further studies with mammals, with a view to obtain unanticipated results regarding the issues mentioned above and therapeutic applications of the RNAi system.

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